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GRANT NUMBER DAMD17-98-1-8187

TITLE: MR Imaging and Gene Therapy of Breast Cancer

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REPORT DATE: July 1999

TYPE OF REPORT: Annual Summary

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Annual Summary (1 Jul 98 - 30 Jun 99)

4. TITLE AND SUBTITLE
MR Imaging and Gene Therapy of Breast Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8187

6. AUTHOR(S)
Min-Ying Su, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California
Irvine, California 92697-1875

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The main purpose of this Career Development Award is for the P.I. to receive training in the field of Cancer Biology and Virology, so that she can apply her expertise, Magnetic Resonance Imaging, for developing non-invasive techniques to monitor gene therapy induced cellular and vascular changes in breast cancer. The P.I. has attended several courses including Virology, Cancer Biology, Molecular Biology and Molecular Biology of Cancer during the initial year of the funding period. She has also learned the protocol of encoding genes into adenovirus. A research assistant has been recruited and is currently undergoing training. They are now working on examination of tissues for various morphological and cellular characteristics. For search of appropriate recombinant viral systems that are expected to cause direct cell killing and indirect killing via microvasculature damage, the P.I. has done extensive literature search. The search results were also used in a proposal submitted to California Breast Cancer Research Program. A plasmid containing VEGF121 has been provided to the P.I. for this project. The transcfection of this gene (sense and anti-sense) into adenovirus are in progress with the support of the UCI Viral Vector Facility. The gene therapy monitoring experiments with MRI will start as soon as the virus is available.

14. SUBJECT TERMS
Breast Cancer
Gene Therapy, Angiogenesis, MRI, Therapy Monitoring, Adenovirus

15. NUMBER OF PAGES
26

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

NA For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

NA In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

NA In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

NA In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

7/5/99

PI - Signature Date

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(5) Introduction

This training proposal (Career Development Award) has two major goals: 1) Personally for me to go through the necessary training and become an independent breast cancer investigator, and 2) Scientifically to find the non-invasively measurable imaging parameters that reflect the underlying biological changes to predict the eventual efficacy of gene therapy. As a physicist working in the field of medical imaging research for years, I have accumulated sufficient experience in the imaging technology, especially for the application in cancer diagnosis and prognosis. But my training and experience in tumor biology and gene therapy is not adequate. Therefore, to carry out the study, I need to receive training in the application of recombinant virus technology for gene therapy. The training will catalyze my development in the promising field of gene therapy and upgrade my status to an independent investigator.

After gaining sufficient experience, I will choose three appropriate recombinant viral systems to work with. Ideally the three viral systems should work through the mechanisms of direct cell killing, indirect killing via microvasculature damage, and a combination of both, respectively, in achieving the therapeutic effect. MR imaging and biological tissue examination techniques will be combined to study the prognosis of tumors undergoing these different types of gene therapy. The longitudinal structural and vascular changes in tumors will be measured with MR imaging. Also, the associated biological or pathological characteristics of the tumors before and at certain times after the treatment will be measured. The results obtained from the imaging study will be correlated with the underlying biology to investigate their relationships. By working with these different mechanisms, I hope to study the limit and the extent of the information that MRI measurements of cellular volume and vascular changes can provide.

(6) Body

In the proposal I planned to achieve 6 specific aims during the three year funding period:

* 1. Obtain basic training in general tumor biology and standard laboratory work,
* 2. Pursue specific training in the application of recombinant virus technology for gene therapy,
* 3. Choose and, if necessary, develop three appropriate recombinant viral systems that are expected to cause direct cell killing, indirect killing via microvasculature damage, and a combination of both, respectively,
4. Study the longitudinal structural and vascular changes in tumors receiving the three viruses with MRI,
5. Study the associated changes in the biological or pathological characteristics of the tumors at various times after the treatment,
6. Correlate the changes in the MRI parameters with the underlying biological changes to establish the relationships between them.

"*" on-going projects in Yr-01

I have been working on Aims 1-3 during the Yr-01 of the project period. In the proposal it was stated that " As the first step I will need to increase my knowledge, through auditing courses and reading books and scientific papers. The critical courses include "Principles of Cancer Biology", "Biosynthesis of Nucleic Acids", "Structure and Biosynthesis of Proteins", and "Virology", which are offered in the Department of Molecular Biology and Biochemistry." For the course training I have audited the following courses:

Dept.	Course No.	Title	Instructor
Bio Sci	124	Virology	Wagner E.
Bio Sci	217B	Cancer Biology II	Fan H.
Bio Sci	99	Molecular Biology	Manning J.E. Hamkalo B.H. Luecke H.
Bio Sci	125	Molecular Biology of Cancer	Fan H.

For Aim 2, I have learned the protocol of encoding the genes into adenovirus. I have also recruited a research assistant, Jun Wang, who is now undergoing training for the cell culture techniques and the animal handling techniques. We will start to work on examination of tissues using fixed or frozen sections.

For Aim 3 I have been working on searching the appropriate systems for the proposed study, i.e. to find genes that can be encoded into AD to reach therapeutic effect by direct cell killing and inhibition of angiogenesis. I have done extensive literature search, and have included the results in the proposal submitted to California Breast Cancer Research Program. The search results are summarized here.

Angiogenic and Anti-angiogenic Gene Therapy Using Adenovirus Systems

A new application of this recombinant virus gene therapy that targets the angiogenesis of cancer is also emerging. Previous cancer therapy design was based on two basic mechanisms: direct cell killing and indirect killing via nutrient depletion by causing microvasculature damage. Although the latter has not been of great interest in main stream research of anti-cancer gene therapy, it is worthy of more attention. Among the 4 viral systems, adenovirus seems to be the most promising one to express the genes for the angiogenic or anti-angiogenic therapy.

Angiogenic gene therapy has been shown as a promising treatment for ischemic disorders [29-32]. Meuhlhauser et al. constructed a replication-deficient adenovirus Ad vector AdCMV.VEGF165 containing the cDNA for human VEGF165, a secreted endothelial cell-specific angiogenic growth factor [29]. The human umbilical vein endothelial cells (HUVECs) infected with the virus exhibited VEGF mRNA expression and protein. When the AdCMV.VEGF165 was injected subcutaneously into mice, histological evidence of neovascularization in the tissues surrounding the injection site was found. Magovern et al. used direct myocardial injections of adenovirus vectors carrying the DNA for the angiogenic protein vascular endothelial growth factor for treatment of myocardial ischemia [30]. Localized expression of vascular endothelial growth factor was achieved for up to 7 days after a single vector administration. Using a hindlimb ischemia model in rats and a myocardial ischemia model in swine, Mack et al. further demonstrated that local administration of a replication-deficient adenovirus vector expressing complementary DNA for VEGF (AdVEGF) would induce collateral vessel formation that could protect against subsequent acute vascular occlusion [31-32]. They concluded that adenovirus-mediated direct myocardial gene transfer can be accomplished safely, providing high levels of protein expression in a greater spatial distribution than previously reported, with minimal transfection of distant organs. Adenovirus vectors coding another family of angiogenic factors, fibroblast growth factor (FGF), have also been shown as a new approach to stimulate angiogenesis in vivo for treatment of ischemic diseases [33-35].

Anti-angiogenic therapy has become increasingly important for cancer treatment in the last decade. Adenovirus could be used to mediate expression of genes that induce anti-angiogenic activities. These genes include a secreted form of the extracellular domain of the flt-1 VEGF receptor, wt p53 tumor suppressor genes which down-regulates vascular endothelial growth factor (VEGF) expression, and Tie2 (a.k.a Tek) endothelium-specific receptor tyrosine kinase known to play a role in tumor angiogenesis [36-38]. Kong et al. constructed an adenovirus (Ad) vectors to mediate transfer of a secreted form of the extracellular domain of the flt-1 VEGF receptor (Adsflt), and demonstrated that Adsflt would suppress tumor growth and metastasis on a regional basis [36]. They concluded that Ad-mediated in vivo regional delivery of the extracellular domain of the flt-1 VEGF receptor may provide a means to control tumor growth within the treated organ without the risk of systemic antiangiogenesis. Bouvet et al. transfected two human cancer cell lines with p53 mutations, with a replication-defective adenoviral vector containing the wild-type p53 gene (Ad5/CMV/p53) [37]. Transduction of cancer cells with wild-type p53 decreased VEGF RNA expression compared with that of controls, thus demonstrated that Ad5/CMV/p53 could inhibit tumor cell-induced angiogenesis in vivo. Lin et al constructed an adenoviral vector to deliver a recombinant, soluble Tie2 receptor AdExTek to block the Tie2 activation [38]. They demonstrated that by blocking the Tie2 pathway both the tumor growth and metastasis were inhibited. These evidences support that recombinant Ad virus system may play a very important role in the anti-angiogenic cancer treatment in the near future.

I have also obtained a plasmid containing the VEGF 121 gene (Vascular Endothelial Growth Factor), from Dr. A. Harris at the University of Oxford, UK. We are currently in the process of purifying it, and later we will transfect the gene into adenovirus. This vector is expected to enhance the angiogenesis of tumor. Then we will reverse the gene to synthesize an adenovirus containing anti-sense VEGF. We will use this Ad containing sense and anti-sense VEGF for therapy study proposed in aims 4-5.

(7) Appendix

1) A bulleted list of key research accomplishments:

- Audit several courses including virology, cancer biology, molecular biology and molecular biology of cancer
- Recruited a research assistant and started training him for cell culture, animal work, and tissue preparation techniques
- Did extensive literature search to study the current development in application of gene therapy technique for therapy
- Obtain a plasmid containing VEGF to transfect the gene into adenovirus

2) A list of reportable outcomes

- One abstract submitted to the ISMRM "International Society of Magnetic Resonance in Medicine" has been accepted for oral presentation, enclosed herein. "Prediction of Gene Therapy Induced Volumetric Changes by Intravascular Volume Changes Measured Using Dynamic Contrast Enhanced MRI."

- One manuscript entitled "Gene Therapy Induced Tumor Size Changes Predicted by the Vascularity Changes Measured Using Dynamic Contrast Enhanced MRI" has been prepared. Although it is not in the final form yet, the current version is enclosed.

- Submit a proposal entitled " Synthesis of a Detectable Neovasculature Targeted Adenovirus" to California BCRP

3) A copy of each of the above cited manuscript and abstract
One ISMRM abstract:
M-Y. Su, J. A. Taylor, L. P. Villarreal and O. Nalcioglu, Prediction of Gene Therapy Induced Volumetric Changes by Intravascular Volume Changes Measured Using Dynamic Contrast Enhanced MRI. in "Proc., 7th ISMRM Annual Meeting, Philadelphia, USA, 1999" p145.

One manuscript
J. A. Taylor, M.-Y. Su, O. Nalcioglu, and L. P. Villarreal "Gene Therapy Induced Tumor Size Changes Predicted by the Vascularity Changes Measured Using Dynamic Contrast Enhanced MRI" has been prepared.

Prediction of Gene Therapy Induced Volumetric Changes by Intravascular Volume Changes Measured Using Dynamic Contrast Enhanced MRI

Min-Ying Su, Jason A. Taylor[1], Luis P. Villarreal[1], and Orhan Nalcioglu
Health Sciences Research Imaging Center and [1]Department of Molecular Biology, University of California, Irvine, CA 92697

Purpose

As new advances in immunotherapy in cancer treatment emerge, there is an ever increasing need to determine the efficacy of therapy as early as possible in order to optimize treatment regiments. However, current methods for assessment are limited to standard imaging techniques, or to invasive tumor biopsies. It would be extremely useful to develop a non-invasive technique to predict treatment efficacy prior to changes in tumor growth. In this study we used a bilateral allogenic rat tumor model, treated it with three recombinant viruses expressing various genes: mouse interferon gamma (IFN-γ), mouse interleukin 1 alpha (IL1-α) and human transforming growth factor-ß (TGF-β), and monitored the volumetric changes after the treatment. The intravascular volume changes were also measured by dynamic contrast enhanced magnetic resonance imaging (MRI). The vascular volume changes were correlated with future volumetric changes in tumors' growth.

Methods

Five Wistar rats were injected with 5 x 10^6 C6 glioma cells bilaterally and subcutaneously into the rear haunch of each rat. A pre-treatment baseline study was first conducted at 2 weeks after tumor implantation. Rats were anaesthetized with im. injection of Ketamine (50mg/Kg) and Rompun (5mg/Kg). All experiments were performed on a 1.5 Tesla General Electric Signa Scanner using a GE linear head coil. The T2-weighted images covering across the tumor were acquired for volumetric measurements. The contrast agent, Gd-DTPA (0.1 mmol/kg), was injected via the tail vein, and the kinetics were measured with a spin-echo T_1-weighted imaging sequence. The kinetics were analyzed with a 2-compartmental pharmacokinetic model to measure the vascular volume.

The incompetent human recombinant adenovirus 5, containing the exogenous genes driven by the human cytomegalovirus immediate early promoter, were constructed. Briefly, the cDNA for the genes encoding for mouse interferon gamma (IFN-γ), mouse interleukin 1 alpha (IL1-α) and human transforming growth factor-ß (TGF-β), were obtained from ATCC and cloned into the polylinker of the plad packaging vector and subsequently co-transfected into 293 cells with PJM17 to obtain recombinant virus. Three animals received left-sided intratumoral injection of 2 x 10^8 plaque forming units of virus, one from each kind of virus, within 24 hours after baseline measurement were taken. Two animals received saline as controls. The post-treatment experiments were repeated at 4, 7, 11, and 15 days after the baseline studies. The longitudinal volumetric changes and the changes in the intravascular volumes were calculated.

Results

We used a subcutaneous bilateral allogenic rat tumor model to evaluate the effectiveness of MRI assessment of tumor growth and regression. Volumetric measurements were taken at day: 0 (baseline), 4, 7, 11, and 15. The tumors in the control rats continued to grow between baseline and day 4, increasing in size by an average of 72%, while the tumors inoculated with recombinant human transforming growth factor beta (rTGF-β) and with recombinant mouse interferon gamma (rIFN-γ) rats to regress. The recombinant mouse interleukin alpha (rIL1-α) inoculated rat continued to grow over the same period, increasing in size by an average of 140%. By day 7, control tumors began to regress (-40%), and the rTGF-β and rIFN-γ infected tumors disappeared, while the tumors in the rIL1-α infected mouse continued to grow by an average of 159%. Though only the right tumor was injected, biological effects were seen bilaterally demonstrating that the effects of the recombinant cytokines infections were systemic and not localized to the site of injection.

The vascular volume was measured from the kinetics of Gd-DTPA in each tumor at different time points. Though the tumor size in both control and rIL1-α infected animals increased between baseline and day 4, the vascular volume of control tumors decreased by 62% (compared to baseline), while the rIL1-α inoculated animal had an increase in the tumor's vascular volume of 41%. These changes of vascular volume were predictive of tumor growth. At day 7 control tumor had decreased by 40% while the rIL-1α infected animal tumors increase by 159%. At day 7 the vascular volume of the rIL1-α animal tumors decreased, and the vascular volume of the control tumors continued to decrease. Again these changes were predictive of tumor growth. Tumor size of the rIL1-α animal did not increase as much as previously at day 11 (+40%), while the tumor continued to regress in the control animals (- 80%). The TGF-β and IFN-γ treated tumors almost disappeared at day 4. The tumor size was too small for the vascular volume measurement. Since the effect was not site dependent, the results of the two tumors in each animal were averaged. The results are summarized in table 1.

	Volumetric Change		Vb Change
	Day 4/Day 0	Day 7/Day 4	Day 4/Day 0
Control	+ 72 %	- 40 % *	- 62 % *
IL1-α	+ 140%	+ 159% *	+ 41 % *
TGF-β	- 80 %	- 83 %	NA
IFN-γ	- 94 %	- 100 %	NA

Table 1. The percentage changes of tumor sizes and the intravascular volumes (Vb) in the control and gene therapy treated tumors. * Note that the vascular volume change of (Day 4/ Day 0) predicts the volumetric change of (Day 7/Day 4).

Discussion

MR imaging is a non-invasive technique that has been used to assess tumor growth. However the ability of using MRI to measure dynamic parameters has not been extensively utilized in a clinical setting. In this study we demonstrate that the ratio of vascular volume, determined using dynamic contrast-enhanced MRI, is predictive of future tumor growth. In all 6 tumors (control and IL1-α treated), which were increasing in tumor size at day 4, a decrease in tumor size several days later was preceded by a decrease in vascular volume at that time. The predictive value was valid for both unmodulated allogenic tumor rejection (4 untreated tumors) and when there is modulation of an immune response (2 tumors from the rIL1-α treated animal) resulting in a delayed onset of tumor rejection. Immune modulation that increased the immune response (rTGF-β and rIFN-γ) resulted in an accelerated tumor rejection which prevented the evaluation of vascular volume as a predictive tool. This technique can potentially be used to assess the efficacy of immunological and other gene therapy treatments, at very early time points, allowing for therapeutic modulation prior to onset of tumor growth changes.

Acknowledgement

This work was supported in part by the California BCRP grants numbered 1RB-0160 and 3IB-0028, and the US ARMY grant numbered DAMD17-98-1-8187.

Gene Therapy Induced Tumor Size Changes Predicted by the Vascularity Changes Measured Using Dynamic Contrast Enhanced MRI

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Using dynamic contrast enhanced magnetic resonance imaging, we demonstrate that the measurement of vascular volume changes is predictive of tumor regression. This method was illustrated utilizing a spontaneously regressing allogeneic rat tumor model. In untreated animals, tumor regression was preceded by several days with a decrease in vascular volume. When the tumor regression was perturbed in an animal via intratumoral injection of recombinant adenovirus expressing human IL-1_, the delayed onset of tumor regression was again preceded by a decrease in vascular volume. The ability to non-invasively determine the efficacy of gene therapy in individuals prior to changes in tumor growth would represent a powerful oncologic tool.

Introduction

As new advances in immunotherapy in cancer treatment emerge, there is an ever increasing need to determine the efficacy of therapy as early as possible in order to optimize treatment regimens. Unfortunately, the conventional imaging techniques determine tumor size but do not predict future tumor size increase. In the last few years there has been increased evidence of the role of angiogenesis and vasculalization on tumor growth (for a review see #@$). However the prevailing methods assessing the level of angiogenesis, which involve analyzing tumor biopsies, are both invasive and time consuming and thus is not very practical. Thus it would be extremely useful to develop a non-invasive technique to predict treatment efficacy prior to changes in tumor growth.

Using a bilateral allogeneic rat tumor model, we demonstrated that vascular volume changes, measured by dynamic contrast enhanced magnetic resonance imaging (MRI), correlated with future changes in tumor growth. Conventional MRI utilizes the high T_2 relaxation times of tumors (associated with high water content) to detect suspicious tumors. However, the edematous or necrotic regions that are also with high water content can not be differentiated from the viable tumor regions, and the spatially resolved vascular characteristics within the tumors can not be revealed from the T_2-weighted images. Dynamic contrast enhanced MR imaging is a non-invasive technique that can be used to measure the time-varying distribution of contrast agents within tissues, from which the information of vascular volume and permeability can be derived [see review (1)]. Vascular volume and permeability are two important properties in diagnosis and prognosis of a tumor. They have been shown to achieve some success in differentiating between benign and malignant tumors [see review (2)] and in determining the aggressiveness of tumor growth (2-4). They were also sensitive to chemotherapy and radiation therapy (5-9), thus may be used to predict the treatment outcome. Gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA, with a molecular weight of 0.57 kD) is one of the most commonly used contrast agents in MRI. After intravenous injection of a contrast agent, it initially occupies the vascular space, and then diffuses into the interstitial space. The amount of agents in the vascular space is related to vascular

volume, and the amount of agents in the interstitial space is related to the vascular permeability and the interstitial space volume. When the agent is mixed into blood or leaked into the interstitial space, the Gd ion shortens the T_1 relaxation time and increases the signal intensity in the T_1-weighted images. Therefore by monitoring the signal intensities in the T_1-weighted images acquired dynamically after injection of contrast agents, the kinetics of the agent within a tumor can be measured.

Materials and Methods

Establishment of an allogenic bilateral rat tumor model. All procedures were in accordance with protocols approved by the University Institutional Animal Care and Use Committee. Wistar rats (obtained from Charles River [Wilmington, MA]) were injected with 5 x 10^6 C6 cells bilaterally and subcutaneously into the rear haunch of each rat. C6 cells are a *N*-nitrosomethylurea-induced glial tumor line derived from BDIX mice (10), and were obtained from American Type Culture Collection (ATCC [Manassas, VA]). In our laboratory, tumors grew in Wistar rats for 3 weeks before they slowly spontaneously regress.

MRI measurement procedure. Rats were anaesthetized with an intramuscular injection of Ketamine (50mg/Kg) (Phoenix Pharmaceutical Inc., [St. Joseph, Missouri]) and Rompun (5mg/Kg) (Lloyd Laboratories, [Shenandoah, Iowa]). Rats were immobilized on a board to prevent incidental movement. A tail vein intravenous line was then established, using a 25 gauge butterfly syringe (Becton Dickinson, [Sandy, Utah]), prior to imaging. All experiments were performed on a 1.5 Tesla General Electric Signa Scanner using a GE linear head coil. Once positioned in the scanner, alignment markings were placed on the center of the tumor in each animal to ensure the same positioning in the subsequent follow-up studies. A set of T_2-weighted images (3 mm thick each) covering the whole tumor were acquired to measure the tumor volume. Then a slice (5 mm thick) through the center of the tumor was prescribed for the dynamic T_1-weighted MRI study. T_1-weighted dynamic contrast-enhanced imaging was performed using the

SE pulse sequence with a (repeat time)/(Echo time) = (100)/(14) ms with a temporal resolution for each image of 14 s. The contrast agent, Gd-DTPA (0.1 mmol/kg, Magnevist®, [Wayne, NJ]), was injected via the tail vein intravenous line after acquiring 4 pre-contrast T_1-weighted imaging images. The sequence was applied to continuously acquire the post enhanced images for 14 min after injection.

MRI image analysis procedure. For each tumor slice, the region of interest (ROI) was manually outlined from the T2-weighted images acquired across the whole tumor. The total tumor volume was calculated by summing over all outlined regions in the set of T2-weighted images. Then based on the tumor slice selected for dynamic contrast enhanced study, an ROI was outlined. The mean signal intensity of this tumor ROI in each pre and post contrast T1-weighted image was measured. The enhancement was calculated by subtracting the pre-contrast signal intensity (averaged over the 4 pre-contrast images) from the post-contrast signal intensity at every time point. Since the enhancement is approximately proportional to the concentration of the contrast agent, the time course of the enhancement represents the kinetics of Gd-DTPA concentration in the tumor.

The kinetics from the liver were also measured to serve as references. The liver is composed of discontinuous capillaries; the contrast agents can easily diffuse in and out of the vessels and reach equilibrium between these two compartments. Because the equilibrium can be reached quickly, the agent is being distributed in the whole extracellular space in the liver and that the measured kinetics approximates the vascular kinetics of the contrast agent. We have previously developed a pharmacokinetic analysis technique which models the distribution of the contrast agents between intravascular and extravascular compartments (12). After vascular and interstitial contributions are separated, the derived parameters can be used to quantitatively characterize the vascular volume and permeability. The vascular component represents signal generated from within the blood vessels, while the extravascular component is generated from signal derived from the extravascular space. By referencing to the extracellular volume of liver (24%), the separated vascular kinetics can be converted into the fractional vascular volume. By assuming the density of

the blood is approximate 1 (g/ cm^3), the fractional vascular volume was expressed as the volume of blood per mass of tissue (cm^3/g). The details of the procedure have been described in a previous publication (12). The analysis in the follow-up studies followed the same procedure. From the ratios of the vascular volumes taken on separate days longitudinally, relative changes in the vascular volume of the tumors were determined.

Recombinant virus. The construction and propagation of replication incompetent human recombinant adenovirus 5, containing the exogenous genes , driven by the human cytomegalovirus immediate early promoter, were previously described (11). Breifly, the cDNA for the genes encoding for mouse interferon gamma, mouse interleuken 1 alpha and human transforming growth factor-ß, were obtained from ATCC and cloned into the polylinker of the plad packaging vector and subsequently cotransfected into 293 cells with PJM17 to obtain recombinant virus. Using a similarly constructed recombinant virus containing β-galactosidase, C6 cells was shown to support recombinant gene expression both in vitro and in vivo (data not shown). Animals received left-sided intratumoral injection of 2 x 10^8 plaque forming units of virus within 24 hours after baseline measurement were taken.

Results

In this experiment we used a subcutaneous bilateral allogeneic rat tumor model to evaluate longitudinal size and vascular changes using MRI. We took advantage of this allogeneic model to evaluated the effectiveness of MRI assessment of tumor growth and regression.

Confirmation of In-Vitro Gene Expression

In vitro studies testing for recombinant gene expression using recombinant human adenovirus containing the β-galactosidase gene driven with a CMV promoter (rβ-gal). C6 cells were grown in 6 wells plates to 60-80% confluency. At which time media was removed and 200 υl of media containing 10, 1, 0.1 MOI of rβ-gal was overlaid onto each plate and incubated for

one hour. Media was subsequently replaced and the cells were incubated for 37 addition hours. The cells were then washed, fixed and stained using X-gal, a substrate for b-galactosidase gene product (The protocol is on my desk in the lab). Using this technique, 80%, 50%, and 5% of the cultured cells where shown to express the recombinant gene.

In vivo studies: Using a similar technique, rβ-gal was injected intratumorally into pre-established tumors. After 36, 72, and 98 hours, the tumors were extracted, fixed and stained with X-gal. The results showed that there was localized recombinant gene expression at the site of viral injection. MOI: multiplicity of injection. If the dish has $2*10^6$ cells, MOI of 10 means $2*10^7$ viruses were added.

Changes of Tumor Volumes

In order to determine accurate tumor volumes, T_2-weighted imaging was performed on the rats seen in Figure 1. From a set of consecutive slices, the tumor volume can be determined from a series of these images a tumor volume was established. Volumetric measurements were taken at day: 0 (baseline), 4, 7, 10, 14, and 18 (Table 1). The tumors in the control rats (rat A-B) continued to grow between baseline and day 4, increasing in size by an average of 72%, while the tumors inoculated with recombinant human transforming growth factor beta (rTGF-β) (rat D) and with recombinant mouse interferon gamma (rIFN-γ) (rat D) rats to regressed. The recombinant mouse interleuken alpha (rIL-1α) inoculated rat (rat C) continued to grow over the same period, increasing in size by an average of 140%. By day 7, control tumors began to regress, and the rTGF-β and rIFN-γ infected tumors disappeared, while the tumors in the rIL-1α infected mouse continued to grow by an average of 159%. Though only the right tumor was injected, biological effects were seen bilaterally demonstrating that the effects of the recombinant cytokines infections were systemic and not localized to the site of injection.

Changes of Vascular Volumes

In each tumor the enhancement kinetics following the IV injection of Gd-DTPA were measured from the series of T_1-weighted dynamic contrast-enhanced images, Figure 2. The kinetics from the liver were also measured. By referencing to the liver kinetics the separated vascular kinetics from each tumor was converted into vascular volume. Though the tumor size in both control and rIL-1α infected animals increased between baseline and day 4, the vascular volume of control tumors decreased by 55% (compared to baseline), while the rIL-1α inoculated animal had an increase in the tumor's vascular volume of 75%. These changes of vascular volume were predictive of tumor growth. At day 7 control animal tumor size had decreased by 42% while the rIL-1α infected animal tumors increase by 150%. At day 7 the vascular volume of the rIL-1α animal tumors decreased the vascular volume of the control tumors continued to decrease. Again these changes were predictive of tumor growth. Tumor size of the rIL-1α animal did not increase at day 11, while the tumor continued to regress in the control animals.

Discussion:

Cytokine effect etc

The enhancement kinetics of Gd-DTPA were analyzed by a pharmacokinetic model to obtain the vascular volume in the current study. Three major factors determine the kinetics of contrast agents in a tissue: blood perfusion, transport of agents across the vessel wall (via diffusion for the small size agent as Gd-DTPA), and diffusion of agents in the interstitium. Perfusion determines the amount of agents delivered to the tissue by blood, thus it is the first dominant factor determining the initial kinetics of the agent in the tissue. When the perfusion is sufficient, the transport of agent across the vessel wall, which is governed by permeability-surface area product (PS) of the vessel wall, is the second factor determining the early kinetics after injection of contrast agent. In our analysis the initial rise phase in the measured enhancement after contrast agent injection is attributed to vascular distribution. However, since the size of Gd-DTPA is relatively small, it can easily diffuse out of the vessel. Therefore, the vascular space volume obtained from the analysis was actually an "apparent vascular volume", which included the true vascular volume, and the fast leakage volume into the interstitial space. Nevertheless, since the agents were delivered into the tumor by blood perfusion, and the percentage change in each individual tumor was obtained from longitudinal studies, the measured vascular volume change is still a good indicator of the vascularity change (specifically include vascular volume and permeability) occurring in the tumor. We have previous shown that by using a macromolecular agent the vascular volume can be measured more accurately. However, none of the macromolecular agents suitable for tumor imaging has been approved by FDA (Food and Drug Administration) yet.

In this study we demonstrate that the ratio of vascular volume, determining using dynamic contrast-enhanced MRI, is predictive of future tumor growth. In all 6 tumors, which were increasing in tumor size, a decrease in tumor size was preceded by a decrease in vascular volume by several days. The predictive value was valid for both unmodulated allogeneic tumor rejection (4 untreated tumors) and when the there is modulation of an immune response (2 tumors from an rIL-

1α treated animal) resulting in a delayed onset of tumor rejection. Immune modulation that increased the immune response (rTGF-β and rIFN-γ) resulted in an accelerated tumor rejection which prevented the evaluation of vascular volume as a predictive tool.

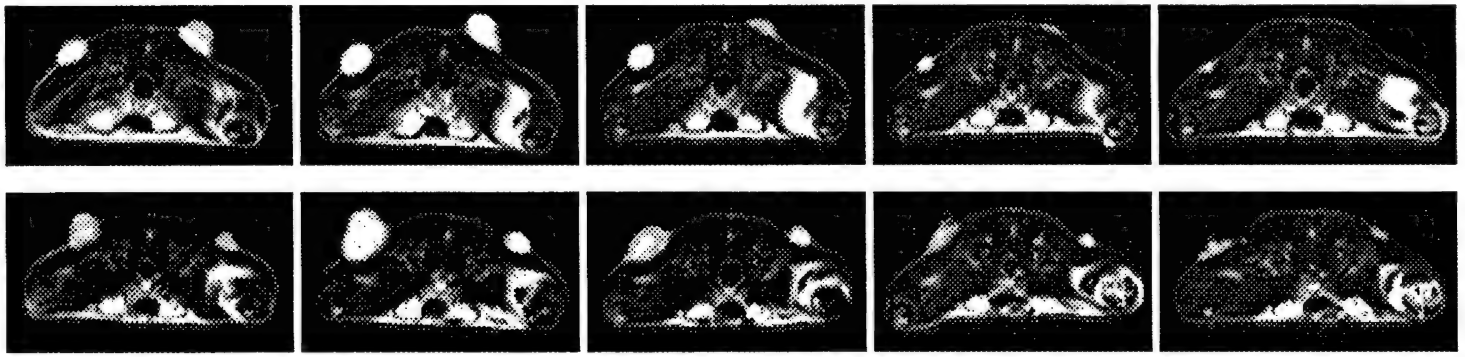
This technique can potentially be used to assess the efficacy of immunological and other gene therapy treatments, at very early time points, allowing for therapeutic modulation prior to onset of tumor growth changes.

Figure 1. A standard T_2-weighted MR images of Wister rats containing subcutaneous bilateral allogeneic tumors. In non-treated animals, tumors grew in size for three weeks prior to the onset of spontaneous regression. Baseline measurements were taken when the average diameter of the tumors reached 0.6 cm, which occurs 14 days post tumor line implantation. Infected animals received 2×10^8 plaque forming units (PFU) of recombinant human adenovirus, containing cytokines as indicated, via an intratumoral injection into the left tumor one day after baseline measurements. **Rat A-B** were non-treated, **Rat C** was inoculated with rIL-1α, **Rat D** was inoculated with rTGF-β, and **Rat E** was inoculated with rIFN-γ.

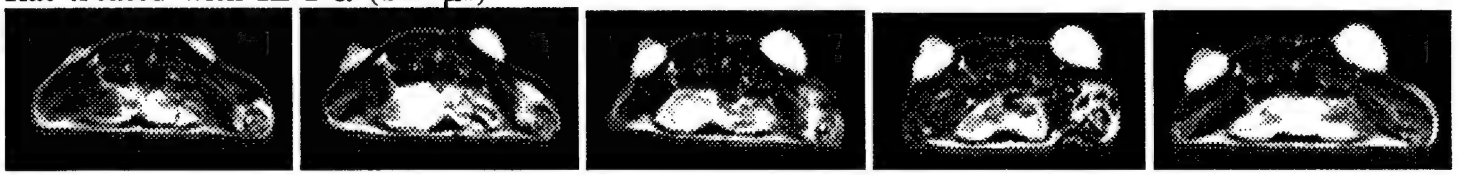
MR images of C6 glioma implanted on the flank of male Wistar rats, untreated and treated with IL-1 α, TNF-β, or IFN-γ
(The bilateral tumors are the bright regions on the flank. The 5 columns show the image of the tumors at 5 different stages)

Baseline day 4 day 7 day 11 day 15

Untreated



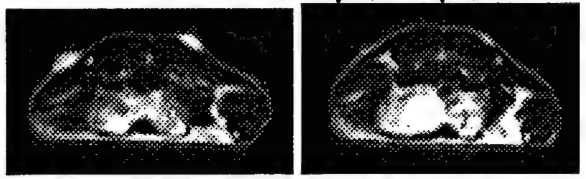
Rat treated with IL-1 α (100 μl)



Rat treated with TNF-β (100 μl)

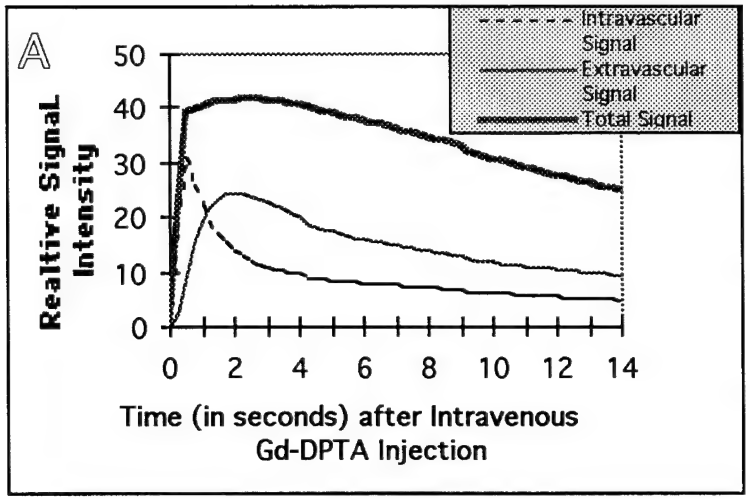
Totally Disappeared

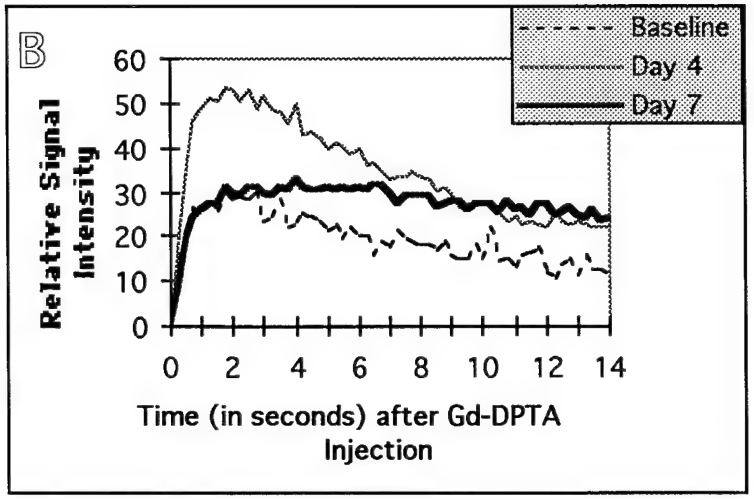
Rat treated with IFN-γ (100 μl)

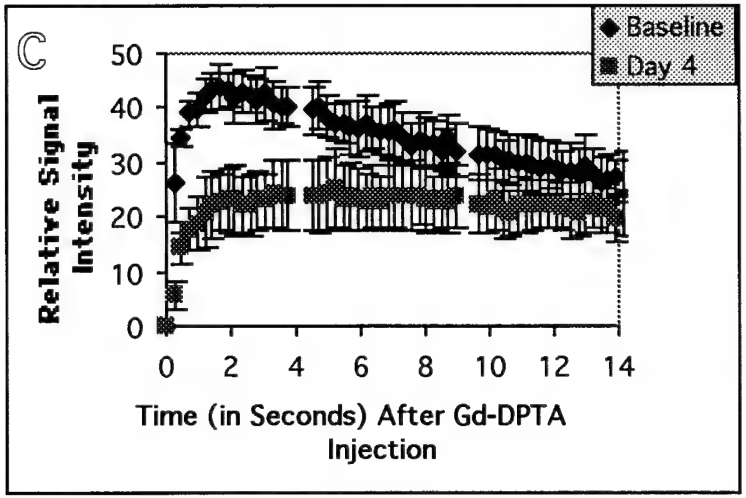


Totally Disappeared

Figure 2. Dynamically acquired change in T_1-weighted signal following GD-DTPA tail vein intravenous injection at time zero for each curve. Panel **A**: baseline curve generated from a control animal tumor. This curve can be broken into intravascular and extravascular components which are shown. Panel **B**: baseline, day 4, and day 7 curves generated from rIL-1α treated rat tumor. Panel **C** and **E** represent the average dynamic change for baseline and day 4 in all control tumors (n=4) and rIL-1α treated tumors (n=2) respectively.


A
Intravascular Signal
Extravascular Signal
Total Signal
Realtive Signal Intensity
50
40
30
20
10
0
0 2 4 6 8 10 12 14
Time (in seconds) after Intravenous Gd-DPTA Injection


B
Baseline
Day 4
Day 7
Relative Signal Intensity
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14
Time (in seconds) after Gd-DPTA Injection


C
Baseline
Day 4
Relative Signal Intensity
50
40
30
20
10
0
0 2 4 6 8 10 12 14
Time (in Seconds) After Gd-DPTA Injection

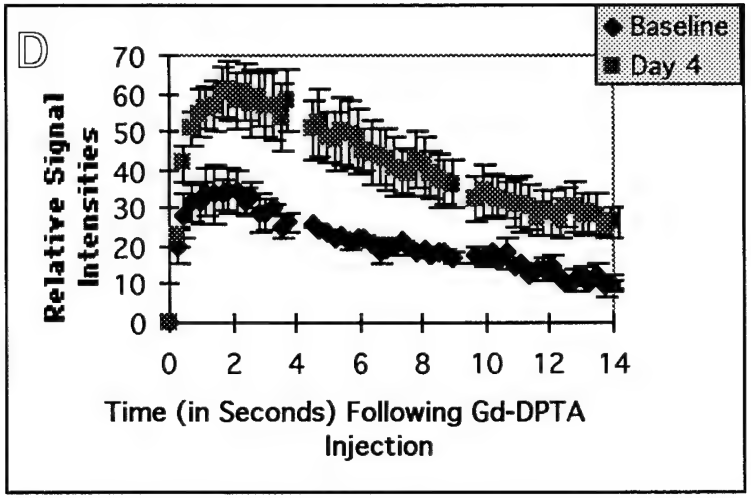

D
Baseline
Day 4
Relative Signal Intensities
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14
Time (in Seconds) Following Gd-DPTA Injection

Table 1. Tumor size differences was determined using reconstructed T2-weighted MR imaging. Serial MRI sections of the rat were taken, and from the analysis of each series, tumor size was determined. Vascular volulume differences were determined using the ratio of intravascular signal curves generated using dynamic contrast-enhansed resonance imaging.

Treatment Groups	Infected Tumors (Side of viral inoculation)		Uninfected Tumors (Side distal to inoculation)		Significance of volume differences between treated and untreated tumors within each treatment group.
	Number of tumors	Tumor Volume ($cm^3 \pm$ SE)	Number of tumors	Tumor Volume ($cm^3 \pm$ SE)	
Control: Mice injected with tumors cells without any viral inoculation	30	1.78 ± 0.55	30	1.84 ± .38	NS
Renca cells and 1.1 x 10^7 PFU MVMp co-injection (MOI of 85)	20	**0.0061 ± 0.0034**	20	**0.67 ± 0.17**	P << 0.001
Renca cells and 1.1 x 10^6 PFU MVMp co-injection (MOI of 8.5)	9[a]	**0 ± 0**	10[a]	**0.35 ± 0.084**	P << 0.004
Renca cells and 1.1 x 10^5 PFU MVMp co-injection (MOI of 0.85)	11[a]	**0.18 ± 0.086**	12[a]	0.78 ± 0.16	P << 0.003
Pre-established Renca tumors injected with 1.1 x 10^7 PFU MVMp	8	0.86 ± 0.40	8	0.77 ± 0.25	NS
Renca cells and 1.1 x 10^6 PFU inactivated MVMp co-injection[b]	15	**0.19 ± 0.10**	15	**0.80 ± 0.23**	P < 0.03

Table 1. Volume comparisons between Renca tumor treatment groups at 30 days post tumor cell inoculation.
Using the bilateral tumor model described in the text, two forms of statistical analyses were preformed. Mean volumes of those with a statistical difference from controls, $P < 0.05$, are highlighted in bold. Statistical differences between infected and uninfected tumors within each treatment condition are shown in the last column. NS = not significant. MOI = multiplicity of infection.

[a] For two conditions there where unequal number of infected versus uninfected tumors. This resulted from the inability to obtain a 30 day volumetric measurement due to ulceration of individual tumors.

[b] Viral inactivation was performed using 2 x 30 minute exposure to a short-wavelength ultraviolet light source. Greater then a 50-100 fold inactivation was confirmed using a dilution cytopathic effects (CPE) assay, where 10-fold serial dilutions of virus were placed onto A92L cells, and the CPE between treated and untreated virus were compared.

Treatment Groups	Infected Tumors (Side of viral inoculation)		Uninfected Tumors (Side distal to inoculation)		Significance of volume differences between the treated and untreated tumors within each treatment group
	Number of tumors	Tumor Volume ($cm^3 \pm$ SE)	Number of tumors	Tumor Volume ($cm^3 \pm$ SE)	
Control: Mice injected with tumors cells without any viral inoculation	12	3.01 ± 0.53	12	2.22 ± 0.45	NS
TSA8 cells and 1.2 x 10^7 PFU MVMp co-injection (MOI of 120)	11	**.031 ± .031**	11	1.35 ± 0.25	P <0.001
TSA8 cells and 1.2 x 10^6 PFU MVMp co-injection (MOI of 12)	5	2.03 ± 0.54	5	2.11 ± 0.52	NS
Pre-established TSA8 tumor injected with 1.2 x 10^7 PFU MVMp	5	2.81 ± 0.56	5	2.73 ± 0.83	NS

Table 2. Volume comparisons between TSA8 tumor treatment groups

Using the bilateral tumor model described in the text, two forms of statistical analyses were preformed. Mean volumes of those with a statistical difference from controls, $P < 0.05$, are highlighted in bold. Statistical differences between infected and uninfected tumors within each treatment condition are shown in the last column. NS = not significant. MOI = multiplicity of infection.

Treatment Groups	Fraction of Renca tumors formed		Fraction of TSA8 tumors formed	
	Infected-Side	Uninfected Side	Infected-Side	Uninfected Side
Control No viral inoculation	---	29/30	---	24/24
Tumor cells inoculated with 2 x 10^7 PFU MVMp	**4/20**	19/20	**1/11**	10/11
Tumor cells co-inoculated with 2 x 10^6 PFU MVMp	**0/10**	10/10	5/5	5/5
Tumor cells co-inoculated with 2 x 10^5 PFU MVMp	**6/12**	11/11	---	---
Tumor cells inoculated with UV inactivated 2 x 10^6 MVMp	**6/15**	13/15	---	---

Table 3. Fraction of tumors formed

Those with a statistically difference from control, at a 5% significance level. Are highlighted in bold. All significant results had a P-value of less then 0.001.